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Immunological Responses to *Salmonella* R Antigens

THE BACTERIAL CELL AND THE PROTEIN EDESTIN AS CARRIERS FOR R OLIGOSACCHARIDE DETERMINANTS

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Summary. Responses in rabbits to heat-killed *Salmonella minnesota* R mutants (chemotypes Ra, Rc and Re) were heterogeneous with respect to the amounts and specific haemagglutinin activities (SHAA) of IgM and IgG antibodies produced to each mutant. Amounts of antibodies in IgM and IgG fractions of sera were determined by quantitative precipitation. For comparison, antibodies were also isolated using an R oligosaccharide-specific immunoadsorbent and quantitated spectrophotometrically. SHAA (haemagglutinating units/mg antibody) of IgG antibodies were similar for all three mutants. In contrast, the Ra mutant induced IgM antibodies with the highest SHAA, while the Re mutant induced IgM antibodies 10-fold lower in activity. The ratio of the amount of IgM/IgG produced was approximately 1/1 for both the Ra and the Rc mutants, while the ratio for the Re mutant was about 1/2. *Salmonella* R oligosaccharide–protein conjugates (chemotypes Rb_2, Rc and Re) were prepared, and the responses to these antigens were compared with those to the heat-killed mutants. The conjugates were specific for the given chemotype, and they were strongly immunogenic when incorporated into Freund's complete adjuvant and administered intramuscularly. Haemagglutinin titres were relatively high, but amounts of antibodies were considerably reduced when the conjugates were administered intravenously without adjuvant. Rabbits immunized with the conjugates in the same manner as with heat-killed R mutants produced predominantly IgM responses in all three cases.

INTRODUCTION

Salmonella R mutants, which contain sequential deficiencies in the R oligosaccharide portion of the lipopolysaccharide molecule (Lüderitz, Westphal, Staub and Nikaido, 1971) represent a series of natural common carrier immunogens (the bacterial cells) with subtle but distinct changes in the structure of the surface antigen determinants (lipopolysaccharides). The R mutant system is therefore an attractive model for studying the immune responses to sequentially modified cell surface antigens.

R mutant oligosaccharides are smaller and less complex in structure than are most O polysaccharides. The immune responses to R antigens of different chemotype are in some ways similar. For example, antibody production in rabbits immunized with heat-killed R

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mutants of chemotypes Ra to Re is comparable in the general shapes of the response curves and in the modes of response to variation in parameters of immunization such as number of injections and dosage of antigen (Kenny and Schlecht, 1971). Also the responses to *Salmonella* R mutants are similar to those directed against various *Salmonella* S forms (Schlecht and Westphal, 1967).

However, the responses to R mutants of different chemotypes show some degree of heterogeneity concerning the haemagglutinin (HA) and precipitin titres, the amount of antibody protein produced and the interrelationship of these values (Kenny and Schlecht, 1971). These differences are not correlated with the chemotype of the mutants tested. The R mutant system thus proved to be a more complex model than had been originally anticipated.

It was recently reported that, in addition to changes in lipopolysaccharide structure, alterations in the outer membrane proteins occur in *Salmonella typhimurium* (Ames, Spudich and Nikaido, 1974) and *Escherichia coli* (Koplow and Goldfine, 1974) R mutants. Changes are also evident in the phospholipid composition of *Salmonella minnesota* R mutants of different chemotypes (Schlecht and Ferber, in preparation). It is therefore possible that the heterogeneity of the responses to R mutants is a reflection of the complexity of the model system with whole bacterial cells as carriers for the oligosaccharide determinants. That is, other cell surface antigens, as well as different amounts or kinds of cellular lipids which could act as natural adjuvants, may have an influence on these responses.

The present investigation was undertaken to determine the nature of the heterogeneities observed. Rabbits were immunized with standard amounts of heat-killed *S. minnesota* R mutants of chemotypes Ra, Rc or Re and the amounts and specific haemagglutinin activities (SHAA) of antibodies in IgM and IgG fractions of the resulting antisera were measured.

In addition, three R oligosaccharides (chemotypes Rb_2, Rc and Re) were prepared by hydrazinolysis of R lipopolysaccharides (Gmeiner, Lüderitz and Westphal, 1969; Gmeiner, Simon and Lüderitz, 1971; Lüderitz, Galanos, Lehmann, Nurminen, Rietschel, Rosenfelder, Simon and Westphal, 1973) and coupled through resulting free amino groups to a common carrier, the protein edestin, via an 'active ester' intermediate (Behrens, Inman and Vannier, 1967; Anderson, Zimmerman and Callahan, 1964). With this series of R oligosaccharide–protein conjugates, effects on the immune responses which might be contributed by the complexity of the carrier could be considered uniform.

The effectiveness of these conjugates was tested in the presence and absence of adjuvants. Responses in rabbits were measured by haemagglutination and by quantitative precipitation. To elucidate further the nature of the heterogeneity of the responses to R mutants, the amounts of IgM and IgG antibodies produced in response to these oligosaccharide–protein conjugates were compared with those formed against heat-killed R mutants as described above.

MATERIALS AND METHODS

Bacterial strains

The R mutants of *Salmonella minnesota* used in this investigation are listed in Table 1. Bacteria were cultivated in a fermenter using the batch technique as previously described (Ring and Schlecht, 1970). Tests for R character and chemotype (Schlecht and Westphal, 1970; Schmidt, Schlecht, Lüderitz and Westphal, 1969) were performed before and after cultivation.

TABLE 1

LIPOPOLYSACCHARIDE STRUCTURE OF THE *Salmonella minnesota* R MUTANTS EMPLOYED (Lüderitz *et al.*, 1971)

Strain	Chemotype	Schematic structure of R lipopolysaccharides
R 60	Ra	GlcNAc | Glc—Gal—Glc—Hep—Hep—KDO—KDO—Lipid A | | | Gal Hep KDO
R 345	Rb_2	Gal—Glc—Hep—Hep—KDO—KDO—Lipid A | | | Gal Hep KDO
R 5	Rc	Glc—Hep—Hep—KDO—KDO—Lipid A | KDO
R 595	Re	KDO—KDO—Lipid A | KDO

GlcNAc = *N*-acetylglucosamine; Glc = glucose; Gal = galactose; Hep = L-glycero-D-mannoheptose; KDO = 2-keto-3-deoxy-D-manno-octonate.

Preparation of bacteria for immunization

Bacteria of chemotype Ra, Rc or Re were cultivated and prepared for immunization as previously described (Kenny and Schlecht, 1971). Suspensions for immunization were adjusted to 2×10^{10} bacteria/ml with 0·3 per cent NaCl.

Production of R oligosaccharides containing free amino groups by hydrazinolysis

Complete hydrazinolysis of R lipopolysaccharides removes all ester- and amide-bound fatty acids and depolymerizes the molecule, leaving the R core oligosaccharide intact, attached to the glucosamine backbone of lipid A (Gmeiner *et al.*, 1969; Lüderitz *et al.*, 1973).

Lipopolysaccharide was extracted from chemotypes Rb_2, Rc and Re (Galanos, Lüderitz and Westphal, 1969) and 600 mg of each was dried overnight at 70° under vacuum. Approximately 6·0 ml of water-free hydrazine (Serva, Heidelberg) were added and allowed to react at 100° for 10–12 hours. The product was then precipitated with cold acetone and washed four times with acetone, once with ethanol and twice again with acetone. The product was chromatographed on Sephadex G-100 (Deutsche Pharmacia, Frankfurt) equilibrated with 0·05 M pyridine–acetate buffer, pH 6·0. Unreacted lipopolysaccharide eluted with the void volume, while the R oligosaccharide ($LP–NH_2$) eluted much later. The $LP–NH_2$ was electrodialysed by the method of Galanos and Lüderitz (in prepatation) to remove contaminating hydrazine. Analyses on the starting materials and products during purification showed that about one half the amount of phosphate and virtually all amide- and ester-bound fatty acids were removed by hydrazinolysis. Electrodialysis removed loosely bound free amino groups (final content = 1·5 moles NH_2/2·0 moles glucosamine) while the molar quantities of glucosamine, ketodeoxyoctonate (KDO) and heptose remained constant.

Preparation of R oligosaccharide–protein conjugates

A hundred and fifty milligrams of oligosaccharide containing free amino groups (LP–NH_2) were dissolved in water-free dimethylsulphoxide (Merck, Darmstadt) containing 200 μl of triethylamine (Serva, Heidelberg). Three hundred and ninety milligrams of the *N*-hydroxysuccinimide ester of *p*-nitrophenylbutyric acid or 'active ester', which reacts readily with the amino groups (Behrens *et al.*, 1967), were added and allowed to react at room temperature for 2 days. The dimethylsulphoxide was then removed by flash evaporation, acetone was added and the resulting precipitate was washed three to four times with acetone. The degrees of coupling for the Rb_2, the Rc and the Re derivatives as determined by free amino group analyses (Ghuysen and Strominger, 1963) before and after reaction with the 'active ester' were 74·3, 89·9 and 47·7 per cent, respectively.

The product (*p*-nitrophenylbutyryl–NH–LP derivative) was reduced with sodium dithionite (Merck, Darmstadt) at 50° for 15 minutes and then chromatographed on Sephadex G-10 equilibrated with 0·2 M ammonium acetate buffer. The fraction eluting with the void volume (*p*-aminophenylbutyryl–NH–LP derivative) was flash evaporated, lyophilized, reconstituted to approximately 9·0 ml with twice distilled water and electrodialysed to remove last traces of buffer. Spectral analyses indicated that reduction was complete in all cases.

The reduced product was diazotized and coupled to edestin (Serva, Heidelberg) according to Himmelspach and Wrede (1971). The resulting conjugate was concentrated by negative pressure dialysis and dialysed extensively against 0·15 M NaCl.

Tests for antigenic specificity of the conjugates

The oligosaccharide–protein conjugates were tested for antigenic specificity by the ability to inhibit haemagglutination systems of various R mutant chemotypes using antisera produced against heat-killed R mutants (Kenny and Schlecht, 1971; Nixdorff and Schlecht, 1972).

Antibody production to heat-killed bacteria and to oligosaccharide–protein conjugates

New Zealand white rabbits (breeding stock of this institute) weighing 2–3 kg were given three intravenous (i.v.) injections of heat-killed bacteria (2×10^{10}/ml) on days 1, 6 and 11 in graded volumes of 0·25, 0·5 and 1·0 ml, respectively. Blood was collected on day 15, shown previously (Kenny and Schlecht, 1971) to be at the peak of both the haemagglutinin and precipitin responses. Experimental details for immunization with the Rb_2, Rc or Re conjugates are presented in the text.

Haemagglutinin assay

Haemagglutinin (HA) titres were determined using Microtiter^R apparatus (Cooke Engineering Company, Alexandria, Virginia) as previously described (Kenny and Schlecht, 1971). HA titres (haemagglutinating units) are reported as the reciprocal of the last serum dilution after addition of antigen to show haemagglutination.

Antibody protein determination

Antibody content of antisera, immunoglobulin (Ig) fractions or fractions isolated from immunoadsorbent columns was determined by a microprecipitation method (Lüderitz, Risse, Schulte-Holthausen, Strominger, Sutherland and Westphal, 1965) using either lipopolysaccharide (LPS) or alkali-treated LPS (LP-Na) as precipitating antigen.

Determination of specific haemagglutinin activities (SHAA)

Serum globulins were precipitated from 6·0 ml of serum with ammonium sulphate (Merck, Darmstadt) at 37 per cent saturation. The globulins were then separated into IgM and IgG fractions on a 3·0 × 100 cm column of Sephadex G-200 equilibrated with phosphate-buffered saline (PBS) (0·05 M Na_2HPO_4/KH_2PO_4 + 0·15 M NaCl + 0·02 per cent NaN_3, pH 7·3). Haemagglutinin titres and concentrations of precipitating antibodies were measured in the pooled, concentrated fractions. From these values the specific haemagglutinin activities were calculated and reported as HA units/mg of antibody.

Preparation of an immunoadsorbent with Rc oligosaccharide specificity

Sepharose 4B (Deutsche Pharmacia, Frankfurt) was activated with cyanogen bromide (Merck, Darmstadt) (Axen and Ernback, 1971; Cuatrecasas, 1970). LP–NH_2 derived from Rc was added and allowed to react at pH 9·8 for 24 hours at 4°. The immunoadsorbent was extensively washed with distilled water and with 0·1 M $NaHCO_3$, pH 9·5. According to dry weight determinations and KDO analyses, the immunoadsorbent contained 120–150 mg of LP–NH_2/gram. After two cycles of adsorption and elution, the amount of LP–NH_2 had not changed.

Adsorption and elution of antibodies on immunoadsorbent columns

Columns 1–2 ml in volume were prepared with the immunoadsorbent in PBS. An Ig fraction was passed through the column, which was then washed thoroughly with PBS. Antibodies were eluted with 2 M sodium thiocyanate (Merck, Darmstadt) in PBS, pH 6·0 (Dandliker, Alonso, de Saussure, Kierszenbaum, Levison and Shapiro, 1967). The eluted antibodies were dialysed overnight against PBS and then concentrated by negative pressure dialysis.

Chemical analyses

2-Keto-3-deoxy-D-manno-octonate (KDO) was determined according to Waravdekar and Saslaw (1959). Glucosamine was measured as described by Strominger, Park and Thompson (1959). Total phosphorus was determined by the method of Lowry, Roberts, Leiner, Wu and Farr (1954). Heptose was measured according to Osborn (1963). Fatty acids were estimated by gas-liquid chromatography of the methyl esters (Weckesser Drews and Fromme, 1972). Free amino groups were determined by the dinitrofluorobenzene method of Ghuysen and Strominger (1963). Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

RESULTS

(1) EFFECT OF ALKALI TREATMENT OF LIPOPOLYSACCHARIDE (LPS) ON ANTIBODY PROTEIN DETERMINATION IN IgM AND IgG FRACTIONS

One of the problems encountered early in this investigation was the determination of the amount of antibody in immunoglobulin (Ig) fractions by quantitative precipitation. This is illustrated in Fig. 1. The figure presents precipitin curves of antibodies in IgM and IgG fractions of antisera produced to heat-killed Rc mutants, measured with either LPS (Fig. 1a) or alkali-treated LPS (LP–Na) (Fig. 1b). The ordinate in both cases represents the total amount of antibody protein in the immunoglobulin fractions, calculated from

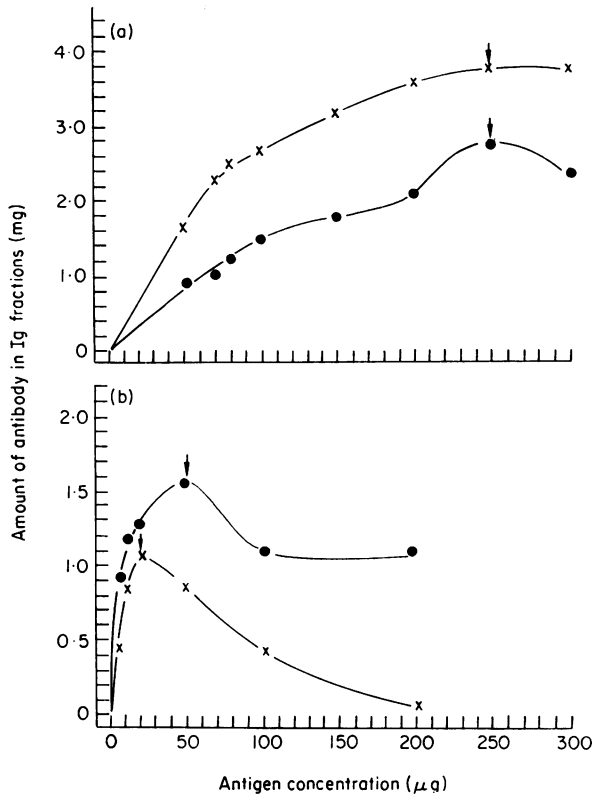


FIG. 1. Precipitation of anti-Rc antibodies in IgM (●) and IgG (×) fractions of antisera (collected on day 15) to heat-killed Rc mutants measured against either (a) lipopolysaccharide (LPS) or (b) alkali-treated LPS (LP-Na). Points on the curves represent the mean of values determined on three separate antisera.

the amount precipitated with the various quantities of antigen added. Each curve represents mean values of determinations on three different antisera.

The curves measured against LPS are in effect absorption curves. With increasing antigen, the amount of IgM and IgG antibodies precipitated increases slowly until a plateau is reached. However, when LPS is solubilized by alkali treatment, the curves look more like true precipitin curves, and equivalence is reached at much lower antigen concentrations (shown by arrows). Differences in the shape of the IgM and the IgG precipitin curves as seen in the bottom graph have been reported for different systems by other investigators (Lindqvist and Bauer, 1966; Bjornson and Michael, 1970).

The concentration of antibodies in Ig fractions determined by precipitation with LP–Na is lower than measurements with LPS, and this has a decided effect on the calculation of specific haemagglutinin activities (HA units/mg of antibody).

For reference, concentrations of antibodies in Ra, Rc and Re antisera, measured by precipitation with LP–Na are 0·4, 1·0 and 1·0 mg/ml, respectively. Haemagglutinin titres of Ra, Rc and Re antisera are 40,000–80,000, 10,000–20,000 and 2500–5000, respectively.

(2) SPECIFIC HAEMAGGLUTININ ACTIVITIES (SHAA) OF ANTIBODIES ISOLATED FROM AN Rc OLIGOSACCHARIDE-SPECIFIC IMMUNOADSORBENT

As an alternative method for determining the concentration of antibodies used in calculating SHAA, we prepared an immunoadsorbent to isolate antibodies from IgM and IgG fractions and measure their concentrations spectrophotometrically. The Rc antigen was chosen as a model for these studies, as relatively large amounts of both IgM and IgG antibodies are produced to this mutant. IgM and IgG fractions from Rc antisera, previously depleted of lipoproteins (Lindqvist and Bauer, 1966), were passed through immunoadsorbent columns as described in the Materials and Methods section. Three measurements were made on the antibody fractions obtained, haemagglutinin titre, absorbance at 280 nm and quantitative precipitation titre with homologous LP–Na. SHAA were calculated from these values.

For comparison, SHAA of IgM and IgG globulin fractions (without passage over immunoadsorbent columns) were measured using antibody protein values determined by precipitation with either LPS or LP–Na.

The results of three different experiments are presented in Table 2. The values for SHAA

TABLE 2

SPECIFIC HAEMAGGLUTININ ACTIVITIES (SHAA)* OF ANTI-Rc IgM AND IgG ANTIBODIES† CALCULATED FROM ANTIBODY PROTEIN CONCENTRATIONS DETERMINED BY VARIOUS MEANS

Rc antiserum	Rc immunoadsorbent batch	Antibody type	SHAA of immunoglobulins (Sephadex G-200) estimated by: Precipitation with LPS‡	SHAA of immunoglobulins (Sephadex G-200) estimated by: Precipitation with LP-Na§	SHAA of antibodies (immunoadsorbent) estimated by: Absorbance at 280 nm	SHAA of antibodies (immunoadsorbent) estimated by: Precipitation with LP–Na§
1	A	IgM	93,000	320,000	200,000	400,000
		IgG	2560	22,000	9000	13,000
2	B	IgM	89,000	440,000	740,000	910,000
		IgG	3400	43,000	47,000	61,000
3	C	IgM	78,000	370,000	527,000	615,000
		IgG	2100	28,000	38,000	47,000

* SHAA = (HA titre)/(milligrams of antibody); HA titre = reciprocal of the last dilution of serum fraction or isolated antibodies to show haemagglutination after addition of antigen.
† Antibodies in IgM and IgG fractions of antisera directed against heat-killed Rc mutants.
‡ Quantitative precipitation with lipopolysaccharide (LPS).
§ Quantitative precipitation with alkali-treated LPS (LP–Na).

determined for Ig fractions by precipitation of antibodies with LP–Na (column 5) agree closely with activities of isolated antibodies calculated either by absorbance at 280 nm (column 6) or by precipitation with LP–Na (last column). These three SHAA values are in turn quite different from activities determined by precipitation of antibodies from Ig fractions with LPS (column 3).

It should be noted that the spectrophotometric estimations of antibody concentration were corrected for any absorption of non-specific protein on the immunoadsorbent column. To accomplish this, we used sera from non-immunized animals, prepared IgM and IgG fractions as described above and absorbed them with Rc bacteria to eliminate any natural antibodies which could react with the Rc antigen. The absorbed IgM and IgG fractions

(same amounts as for Rc antisera) were passed over Rc immunoadsorbent columns identical in batch number and volume as those used to isolate the corresponding fractions from Rc antisera (Table 2). By this method, the amounts of non-specific protein adsorbed varied from about 10–30%, depending upon the immunoadsorbent batch and the fraction of immunoglobulin. These quantities of protein were subtracted from those of the eluted antibodies before calculating SHAA.

In the above experiments, 82–85 per cent of the IgM and the IgG antibodies measured spectrophotometrically at 280 nm were precipitated by LP–Na. Considering the possibility of some inactivation through the elution process (Dandliker *et al.*, 1967), the antibody protein values determined by precipitation with LP-Na and those determined by absorbance at 280 nm were similar.

For the remainder of the investigation, antibody protein concentrations were determined by precipitation with LP–Na.

TABLE 3

AMOUNTS OF ANTIBODIES RECOVERED FROM IgM AND IgG FRACTIONS OF Ra, Rc AND Re ANTISERA* AS DETERMINED BY QUANTITATIVE PRECIPITATION WITH THE HOMOLOGOUS ALKALI TREATED LIPOPOLYSACCHARIDE

Antigen	IgM		IgG		Ratio of IgM/IgG	Total antibody recovered (mg)
	Antibody recovered (mg)	Mean	Antibody recovered (mg)	Mean		
Ra	0·64		0·45			
	0·33	0·49	0·28	0·44	1·11	0·93
	0·50		0·58			
Rc	0·98		0·65			
	1·05	1·31	0·99	1·05	1·25	2·36
	1·90		1·60			
Re	0·83		1·60			
	0·76	0·78	2·40	1·91	0·41	2·69
	0·73		1·75			

* Antisera were produced in rabbits against Ra, Rc and Re heat-killed bacteria. For each mutant tested, measurements were made on three separate antisera.

(3) AMOUNTS OF ANTIBODIES IN IgM AND IgG IMMUNOGLOBULIN FRACTIONS OF Ra, Rc AND Re ANTISERA

Table 3 shows the amounts of antibodies in IgM and IgG fractions of antisera directed against heat-killed Ra, Rc and Re mutants, measured by precipitation with homologous LP–Na. According to these results, the Rc mutant and the Re mutant induced greater amounts of both IgM and IgG antibodies than did the Ra mutant. The ratio of the amount of IgM/IgG was similar for the Ra and the Rc mutants (approximately 1/1), while the Re mutant appeared to induce about twice as much IgG as IgM antibody.

(4) SHAA OF ANTIBODIES IN IgM AND IgG FRACTIONS OF Ra, Rc AND Re ANTISERA

The SHAA of antibodies in IgM and IgG fractions of antisera directed against heat-

killed Ra, Rc and Re mutants are presented in Table 4. In each case the mean values for three different antisera are given (the same antisera as in Table 3). SHAA of antibodies in IgG fractions were similar for all three mutant systems. In contrast, the Ra mutant induced IgM antibodies with high activities while the Re mutant induced IgM antibodies with low activities, almost 10-fold lower than those of the Ra mutant. These differences are reflected in the molar ratios of IgM/IgG.

As to the purity of the IgM and the IgG fractions, immunoelectrophoresis showed that neither fraction was contaminated with IgA and that IgG was free of any detectable IgM. IgM was contaminated with a trace of IgG but this amount was apparently insignificant, as when IgM fractions were purified by re-chromatography on Sephadex G-200, the SHAA did not change. Similarly, the SHAA of IgG fractions further purified by DEAE–cellulose chromatography (Fahey, 1962) were not significantly different.

TABLE 4

SPECIFIC HAEMAGGLUTININ ACTIVITIES (SHAA) OF ANTIBODIES IN IgM AND IgG FRACTIONS OF Ra, Rc AND Re ANTISERA,* CALCULATED FROM ANTIBODY CONCENTRATIONS DETERMINED BY QUANTITATIVE PRECIPITATION WITH THE HOMOLOGOUS ALKALI-TREATED LIPOPOLYSACCHARIDE

	IgM		IgG		Molar ratio SHAA
Antigen	SHAA	Mean	SHAA	Mean	(IgM/IgG)†
Ra	1,000,000		14,500		
	870,000	937,000	32,000	22,000	265/1
	940,000		21,000		
Rc	380,000		20,000		
	420,000	383,000	16,000	23,000	100/1
	348,000		33,000		
Re	128,000		18,500		
	140,000	120,000	32,000	20,000	36/1
	94,000		10,000		

* Antisera were produced in rabbits against Ra, Rc and Re heat-killed bacteria. For each mutant tested, measurements were made on three separate antisera. The antisera are the same as in Table 3.

† Ratio of SHAA of IgM/IgG calculated on a molar basis.

(5) PREPARATION OF R OLIGOSACCHARIDE–PROTEIN CONJUGATES

In order to compare the responses to heat-killed R mutants with those directed against the R oligosaccharides attached to a common carrier, conjugates of three R oligosaccharides (chemotypes Rb_2, Rc and Re) with edestin were prepared, as described in the Materials and Methods section. The Ra conjugate could not be prepared by this method because of the lability of the *N*-acetyl group of the terminal glucosamine to hydrazinolysis.

Free amino group determinations on oligosaccharides before and after reaction with the 'active ester' suggested that coupling took place. Binding was most effective with the Rc LP–NH_2 (90 per cent) and least effective with the Re LP–NH_2 (50 per cent).

In addition, chromatography of the product of this reaction on Sephadex G-10 (Fig. 2) showed that binding took place. *p*-Nitrophenylbutyric acid, which absorbs at 286 nm, eluted in the void volume with the oligosaccharide, which shows no absorption at this wavelength. If acetone washing of the product was omitted, the small peak at fraction 100, which represents unattached *p*-nitrophenylbutyric acid, was much larger.

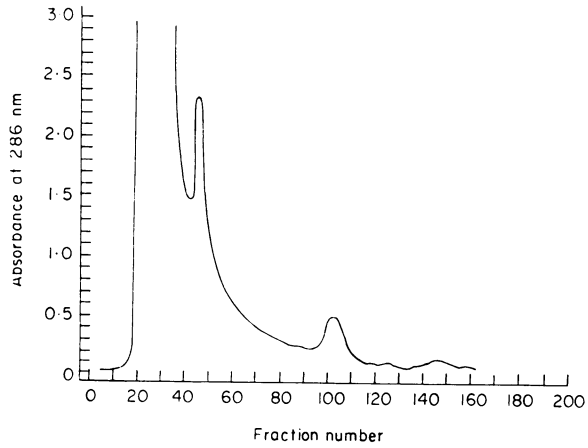
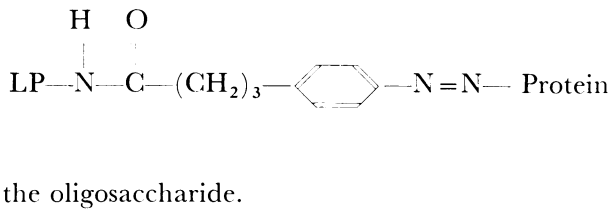


FIG. 2. Sephadex G-10 chromatography of the product of the reaction of R oligosaccharide with 'active ester'. The 1·5 × 100 cm column was equilibrated with 0·2 M phosphate buffer + 0·15 M NaCl pH 7·5. A hundred milligrams of the product, after acetone washing, was placed on the column and eluted with the same buffer.

After diazotization and coupling of the intermediate product to edestin, the degree of substitution of oligosaccharide onto edestin was determined by protein and KDO analyses and expressed as micrograms of LP–NH_2 per milligram of protein. This value did not change significantly after chromatography of the conjugates on Sephadex G-75 equilibrated with 0·2 M NaCl + 0·05 M $NaHCO_3$, pH 9·0, indicating that the oligosaccharide was firmly bound to the protein.

The final product may be represented by the formula



where LP—N— is the oligosaccharide.

(6) ANTIGENIC SPECIFICITY OF THE CONJUGATES

Table 5 presents the results of haemagglutination–inhibition assays used to test the antigenic specificity of the conjugates. In all cases, the R oligosaccharide attached to edestin inhibited only the homologous reaction.

(7) IMMUNIZATION USING THE CONJUGATES WITHOUT ADJUVANT

In order to compare the immune responses to the conjugates with those directed against heat-killed bacteria, rabbits were given three i.v. injections of 2·0, 4·0 and 8·0 mg of either the Rb_2, Rc or Re conjugates on days 1, 6 and 11, respectively. Antisera were collected on day 15. The effect of the degree of oligosaccharide substitution with the Rc conjugate was also tested. Antibody protein concentrations and HA titres are reported in Table 6.

The Rc conjugate with an oligosaccharide substitution of 115 μg/mg edestin was a

TABLE 5

HAEMAGGLUTINATION–INHIBITION TESTS ON *Salmonella minnesota* R ANTISERA* USING EITHER Rb_2, Rc OR Re CONJUGATES† AS INHIBITORS

Inhibitor	Minimal amounts (μg/ml) of LP–NH_2 in conjugate to inhibit HA system of chemotype			
	Ra	Rb_2	Rc	Re
Rb_2 LP–edestin	>2445	⩽1·2	>2445	>2445
Rc LP–edestin	>1400	>1400	2·7	>1400
Re LP–edestin	>2650	n.d.	>2650	41

n.d. = Not determined.

* Antisera were collected from rabbits on day 15 after three intravenous injections (0·25, 0·5 and 1·0 ml) at 5-day intervals of a suspension containing 2×10^{10} heat-killed bacteria/ml. For haemagglutination–inhibition tests, antisera were diluted to contain 3–4 HA units.

† The Rb_2 conjugate contained 489 μg of LP–NH_2/mg of edestin; the Rc conjugate, 200 μg of LP–NH_2/mg of edestin; the Re conjugate, 220 μg of LP–NH_2/mg of edestin.

TABLE 6

ANTIBODY PROTEIN (mg/ml)* AND HAEMAGGLUTININ (HA) TITRE† DETERMINATIONS OF RABBIT ANTISERA TO VARIOUS R OLIGOSACCHARIDE–EDESTIN CONJUGATES‡

Immunogen	Degree of substitution with oligosaccharide (μg/mg of edestin)	Antibody protein* (mg/ml) on day 15	HA titres† on day 15 with lipopolysaccharide of chemotype§					
			Ra	Rb_2	Rc	Rd_1	Rd_2	Re
Rb_2 LP–edestin	489	0·26	16	**500**	16	8	8	2
		0·23	128	**500**	2	2	4	2
Rc LP–edestin	115	0·06	n.d.	n.d.	**640**	n.d.	n.d.	n.d.
		0·03	n.d.	n.d.	**320**	n.d.	n.d.	n.d.
Rc LP–edestin	280	0·12	8	4	**5000**	8	8	8
		0·16	8	8	**5000**	8	8	8
Rc LP–edestin	470	0·16	n.d.	n.d.	**2500**	n.d.	n.d.	n.d.
		0·17	n.d.	n.d.	**5000**	n.d.	n.d.	n.d.
Re LP–edestin	220	0·05	2	4	2	2	2	**64**
		0·03	2	4	4	4	4	**64**

n.d. = Not determined.

* Antibody protein values are reported for two separate antisera in each case, measured using homologous alkali-treated lipopolysaccharide.

† HA titres are the reciprocal of the last serum dilution to show haemagglutination after addition of antigen and are reported for two separate antisera in each case, measured using sheep erythrocytes sensitized with alkali-treated lipopolysaccharide.

‡ For all conjugates, rabbits received three intravenous injections containing 2·0, 4·0 and 8·0 mg (based on weight of edestin) on days 1, 6 and 11, respectively. Sera were collected on day 15.

§ Numbers in heavy type represent homologous HA reactions.

comparatively poor immunogen, inducing antibody protein concentrations of 0·06 and 0·03 mg/ml and homologous HA titres of 640 and 320. Rc conjugates of higher substitution (280 and 470 μg of LP–NH_2/mg of edestin) stimulated the production of higher amounts of antibodies as well as stronger HA titres. However, antibody protein concentrations and HA titres of these antisera were still lower than those of antisera formed against heat-killed R mutants (see Results, section 1), although the total amount of oligosaccharide (3·92 mg) injected with the Rc conjugate (degree of substitution = 280 μg/mg) was about 10-fold more than that injected with the bacteria. Reduction of the dosage of conjugate produced even weaker responses (data not shown).

The immunogenicity of the Rb_2 conjugate with an oligosaccharide substitution of 489 μg/mg edestin was similar to that of the Rc conjugate with a similar degree of substitution, based on amount of antibody protein formed. The Re conjugate, however, induced much poorer responses.

In all cases, the responses were specific for the homologous R lipopolysaccharide. Cross-reactions observed were no greater than those recorded previously for the bacterial immunogens (Nixdorff and Schlecht, 1972).

It should be noted that neither LP–NH_2 nor edestin alone elicited detectable immune responses to LP–Na when administered in the same amounts as found in the conjugates.

(8) IMMUNIZATION USING THE CONJUGATES WITH ADJUVANT

Rabbits were given one (day 1) or two (days 1 and 44) injections (2·0 mg/injection) of the Rc conjugates (oligosaccharide substitutions of 470 and 115 μg/mg of edestin) or the Re conjugate (220 μg of LP–NH_2/mg of edestin) mixed with an equal volume of Freund's complete adjuvant (FCA). The injections (1·0 ml) were given intramuscularly in the four leg regions and subcutaneously in the neck. According to HA titres (Table 7) the responses

TABLE 7

HAEMAGGLUTININ (HA) RESPONSES* OF RABBITS TO Rc OR Re OLIGOSACCHARIDE–EDESTIN CONJUGATES MIXED WITH FREUND'S COMPLETE ADJUVANT (FCA)

Group	Conjugate	Rabbits	Homologous HA titres† measured on day: 1‡	14	28	42	56	63	70	98	116
I	Rc	**442**	<20	160	2500	2500	2500	1280	320	1280	640
	(470)§	**447**	<20	80	2500	2500	320	1280	1280	2500	1280
		326	<20	320	2500	2500	320	160	320	160	320
II	Rc	**443**	<20	<20	1280	1280	1280	1280	1280	2500	640
	(115)§	**323**	<20	<20	320	640	160	160	320	320	640
		445	<20	<20	640	640	640	640	640	640	320
III	Re	**393**	<20	<20	20	40	40	80	80	80	80
	(220)§	**444**	<20	<20	80	<20	320	160	80	160	160
		391	<20	<20	<20	40	80	80	80	80	80

* Two rabbits in each group (numbers in heavy type) were given two injections (2·0 mg each) of the conjugate on days 1 and 44, respectively, in 0·5 ml of saline plus 0·5 ml of Freund's complete adjuvant (FCA). Each of the two 1·0-ml injections was given intramuscularly in the four leg regions and subcutaneously in the neck. One rabbit in each group (roman numbers) was given only one injection (2·0 mg) on day 1 in the same manner.

† Ha titres are reported as the reciprocal of the last serum dilution to show haemagglutination after addition of antigen.

‡ Day 1 gives the HA titre before immunization.

§ Numbers in parentheses represent the degree of oligosaccharide substitution in micrograms per milligram of edestin.

to the Rc conjugate (group I) were the earliest and strongest. Responses to the Re conjugate were detectable later and the titres were much lower. In all three groups, the second injection given on day 44 did not increase titres significantly. In group I the second injection may have helped to keep titres high later in the response, but this was not evident in the other two groups.

Differences in the immunogenicity of these antigens were also apparent when antibody protein contents of the same antisera were measured (Table 8). The concentration of

TABLE 8

ANTIBODY PROTEIN CONTENT OF SERA* FROM RABBITS IMMUNIZED WITH EITHER Rc OR Re OLIGOSACCHARIDE–EDESTIN CONJUGATES MIXED WITH FREUND'S COMPLETE ADJUVANT (FCA)

			Antibody protein content (mg/ml of serum) on day:		
Group	Conjugate	Rabbit	42	63	116
I	Rc	442	1·0	0·96	0·91
	LP–edestin	447	1·0	1·16	0·86
	(470)	326	1·2	0·70	0·80
II	Rc	443	0·50	0·28	0·38
	LP–edestin	323	0·48	0·36	0·26
	(115)	445	0·52	0·44	n.d.
III	Re	393	0·03	0·18	n.d.
	LP–edestin	444	0·14	0·26	0·21
	(220)	391	0·10	0·24	0·23

n.d. = Not determined.

* Sera are the same as in Table 7.

antibodies in sera of group I rabbits (1·0 mg/ml) is comparable to that found in sera of rabbits immunized i.v. with heat-killed Rc mutants (see Results, section 1). The amount of oligosaccharide received by this group (before the second injection) was approximately 2·5 times that received with the bacterial antigen. Again, the immunogenicity of the Re conjugate in group III was considerably lower than that of both Rc conjugates. Also, the second injection did not significantly influence the amount of antibody produced.

The specificity of these responses was tested by HA reactions and quantitative precipitation of antisera with heterologous alkali-treated lipopolysaccharides of *S. minnesota* R mutants. Cross-reactions were no greater than those reported for antisera produced to heat-killed R mutants (Nixdorff and Schlecht, 1972).

(9) AMOUNTS OF IgM AND IgG ANTIBODIES IN ANTISERA TO THE CONJUGATES

The ratios of the amounts of IgM/IgG were determined for antisera to the Rb_2, the Rc and the Re conjugates in the same manner as for antisera to heat-killed R mutants described above, except that globulins were precipitated from 12 ml of serum. Antisera were from rabbits given three i.v. injections of 2·0, 4·0 and 8·0 mg on days 1, 6 and 11, respectively. Rabbits were bled on day 15. The ratios of IgM/IgG for the Rb_2 and the Rc conjugates were 2·2/1 and 2·8/1, respectively. Only IgM could be detected in antisera to the Re conjugate. These results indicate that antibodies produced to all three conjugates after this programme of immunization were predominantly IgM.

When the conjugates were administered i.m. with FCA as described above, the responses were IgG in character: no IgM could be detected in Rc or Re anti-conjugate sera at the height of the responses. This is also reflected in the rather low HA titres as compared to the amounts of antibody produced (Tables 7 and 8).

DISCUSSION

As demonstrated in this report, mutants of *Salmonella minnesota* which have only subtle changes in R oligosaccharide determinant structure induce immune responses in rabbits that are quite different with respect to the amounts and specific haemagglutinin activities (SHAA) of the IgM and the IgG antibodies produced.

Specific haemagglutinin activities of antibodies from immunoglobulin fractions were similar to activities of antibodies isolated and purified by means of immunoadsorbent columns. SHAA calculated using antibody concentrations estimated either by precipitation with alkali-treated lipopolysaccharide (LP–Na) or by protein measurement by absorbance at 280 nm were in good agreement. However, when LPS was used for precipitation, antibody content and SHAA deviated significantly from values mentioned above. The reason for this difference is at present unknown. The amount of non-specific protein absorbed by LPS from sera does not account for the discrepancy.

In addition, IgM and IgG antibodies isolated from the immunoadsorbent were equally efficient in precipitating with LP–Na. Thus, precipitation of antibodies with LP–Na appeared to be a fairly accurate method for estimating the amounts of antibodies to the oligosaccharide determinants in antisera and Ig fractions.

For all three mutants tested, SHAA of IgG antibodies were similar. In contrast, there were remarkable differences in the activities of IgM antibodies (Table 4). These results are reminiscent of a report by Daguillard and Edsall (1968) who studied the activities of isolated rabbit antibodies to the *12* and the *9* O-factors of *Salmonella typhi*. IgG antibodies to these factors had the same specific agglutinating activities, while IgM antibodies to factor *12* were about five times more active than IgM antibodies to factor *9*. The results were best explained on the basis of the greater accessibility of the *12* antigenic determinants on the bacterial surface. It was suggested that the smaller IgG antibodies had equal access to both the *9* and the *12* determinants.

Our results might be explained by a similar analogy. The larger Ra LP–Na molecules on the surface of erythrocytes may be more accessible to anti-Ra IgM antibodies than the Rc or the Re LP–Na molecules to their respective IgM antibodies. Perhaps neighbouring structures on the erythrocyte surface hinder contact between the larger IgM molecules and the antigenic determinants. The heterogeneity in the SHAA of the three systems might therefore be a reflection of accessibility of antigen rather than differences in actual affinities of the antibodies.

The differences in activities of IgM and IgG antibodies are not due simply to a requirement for different amounts of antigen attached to the erythrocyte surface. Fifty micrograms of LP–Na added per 10 ml of a 0·5 per cent erythrocyte suspension was found to be optimal for both IgM and IgG haemagglutinating systems of all three R LPS chemotypes.

The ratios of the amounts of IgM/IgG produced to the Ra, the Rc and the Re mutants were also quite different (Table 3). In contrast, IgM/IgG production to the Rb_2, the Rc and the Re oligosaccharide–protein conjugates was very similar (predominantly IgM when administered i.v. and predominantly IgG when injected i.m. with FCA). These

results suggest that the heterogeneity in the IgM/IgG antibody ratio produced to R mutant bacteria might be due to different properties of the bacterial cells serving as carriers for oligosaccharide determinants.

Oligosaccharide–protein conjugates prepared as described have the advantage that no groups in the antigenic determinant portion of the molecule are involved in the linkage. This is particularly important for R oligosaccharides, especially those of lower chemotype, as they are composed of only a few saccharide units.

Experiments testing the immunogenicity of the conjugates administered i.v. indicated that they were less effective than whole bacteria containing comparable amounts of R oligosaccharide. This is despite the fact that edestin conjugates form visible aggregates at neutral pH which might substitute for the size of the bacterial cell.

In general, conjugates containing higher oligosaccharide/protein ratios (degrees of substitution) were more effective immunogens than those of lower ratios (Tables 6 and 8).

The immunogenicity of the conjugates with regard to production of antibody protein was considerably increased when administered with FCA (compare Tables 6 and 8). The Rc antigen with an oligosaccharide substitution of 470 μg of LP–NH_2/mg of edestin induced amounts of antibodies in rabbits comparable to those produced by heat-killed Rc mutants administered i.v., but the bacteria injected contained a total of about 2·5 times less oligosaccharide.

Regardless of the method of immunization, the Re conjugate was less immunogenic than the Rb_2 or the Rc conjugates. This may either be a reflection of size (Staub, Stirm, Le Minor, Lüderitz and Westphal, 1966; Stellner, Lüderitz, Westphal, Staub, Leluc, Coynault and Le Minor, 1972) or possibly other physicochemical properties of the Re determinant when placed on the artificial carrier. In addition, erythrocytes sensitized with Re antigen were less reactive with IgM antibodies in HA tests than erythrocytes sensitized with Ra or Rc oligosaccharides (Table 4).

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